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Award Number:
W81XWH-08-1-0763

TITLE:
Characterization of IKBKE as a Breast Cancer Oncogene

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REPORT DATE:
October 2010

TYPE OF REPORT:
Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: (Check one)

X Approved for public release; distribution unlimited

☐ Distribution limited to U.S. Government agencies only; report contains proprietary information

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
31-OCT-2010	AnnualSummary	1 Oct 2009 - 30 Sep 2010

4. TITLE AND SUBTITLE
Characterization of IKBKE as a Breast Cancer Oncogene

5a. CONTRACT NUMBER
W81XWH-08-1-0763

5b. GRANT NUMBER
BC083046-PreDoc

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Dana-Farber Cancer Institute
Boston, MA 02115

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research And Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Previous work in the Hahn Lab has identified *IKBKE* as a novel breast cancer oncogene that is capable of human mammary cell transformation. Amplification and overexpression of IKKepsilon was observed in a significant percentage of human breast cancer cell lines and primary tumor samples. Additionally, breast cancer cell lines carrying the *IKBKE* amplicon showed decreased viability in response to IKKepsilon suppression by shRNA. Our work has also demonstrated that IKKepsilon is a non-canonical IKK (IkappaB kinase) family member that activates the NF-kappaB pathway and that this activity is essential for cell transformation. However, it is known that IKKepsilon not does participate in the canonical IKK complex to activate NF-kappaB signaling. Though recent work has sought to identify the downstream targets of IKKepsilon, the mechanism of its upstream regulation is not well-understood. Thus, I propose to further our understanding of IKKepsilon function by investigating the upstream regulation of IKKepsilon – specifically, the role of ubiquitination in IKKepsilon-mediated cell transformation. In addition, I am to investigate the role of IKKepsilon in breast cancer initiation and maintenance *in vivo* through the generation of a constitutive and an inducible transgenic mouse model.

15. SUBJECT TERMS
IKKepsilon, breast cancer, ubiquitin, oncogenic transformation, mammary mouse tumor model

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
U	U	U	UU	9	USAMRMC

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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Introduction

I have made significant progress in both Specific Aims that were originally laid out in my Statement of Work. In this Progress Report, I will report the studies and results that I have conducted over the past two year towards accomplishing those aims.

Specific Aims

1. Investigate the role of ubiquitination in IKKε-mediated cell transformation
    a. Confirm and characterize IKKε ubiquitination in the context of mammary cell transformation
    b. Identification of the IKKε ubiquitin-accepting residues
    c. Determine the functional relevance of IKKε ubiquitination in mammary cell transformation

2. Investigate the role of IKKε in breast cancer initiation and maintenance
    a. Investigate the role of *IKBKE* in breast cancer initiation
    b. Investigate the role of *IKBKE* in breast cancer maintenance

Body: Studies and Results

Specific Aim 1a: I have confirmed that IKKε ubiqitination occurs in a mammary epithelial cell context. I immunoprecipitated IKKε from human mammary epithelial cells that stably overexpress activated MEK (HMECM) along with either Flag-tagged (F-IKKε) or myristolated Flag-tagged (MF-IKKε) IKKε and was able to detect an endogenously ubiquitinated species of MF-IKKε by immunoblotting for ubiquitin (Figure 1A). In parallel, I transiently transfected the same cells with HA-tagged ubiquitin, performed an immunoprecipitation for IKKε and was able to successfully detect an ubiquitinated ladder of MF-IKKε by immunoblotting for HA. I performed the reverse IP/Western experiment, and was also able to immunoblot for IKKε after immunoprecipitation by HA antibody (Figure 1B). Interestingly, I was only able to confirm IKKε ubiquitination in the MF-IKKε transformed HMECM cells and not in the F-IKKε transformed cells. Previous characterization of these cell lines has shown that MF-IKKε exhibits a much more robust transformation phenotype in HMECM cells than the F-IKKε counterpart. Perhaps this variation in transformation phenotype is related to the ubiquitination status of IKKε in these cells.

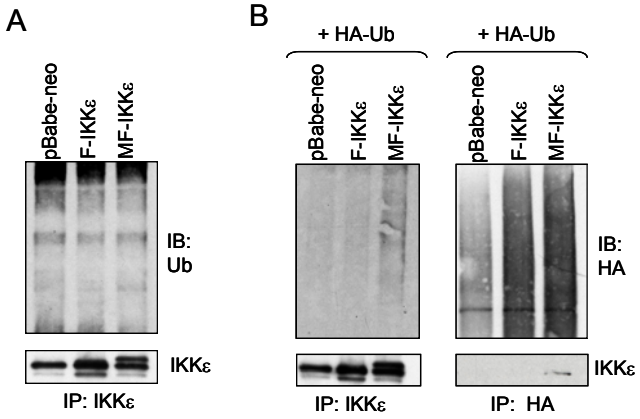
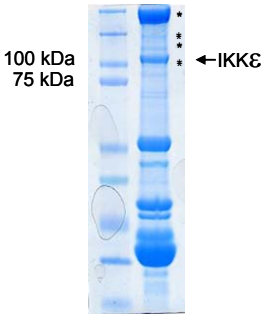


Figure 1. IKKε is ubiquitinated HMECM cells.
(A) Lysates from HMECM cells expressing either pBn, F-IKKε or MF-IKKε. were immunoprecipitated by the IKKε antibody and immunoblotted for ubiquitin.
(B) HMECM F-IKKε or MF-IKKε. cells were transfected with HA-Ub. Lysates were immunoprecipitated by IKKε or HA antibody and then immunoblotted for the other protein.

Specific Aim 1b: I have successfully identified three lysine residues in IKKε that are subject to ubiquitination by mass spectrometry. I transiently cotransfected GST-tagged IKKε and HA-tagged ubiquitin in HEK293T cells and performed a GST immunoprecipitation. The immunoprecipitates were then subjected to SDS-PAGE followed by Coomassie blue staining. Four bands of interest were identified and submitted for mass spectrometry analysis (Figure 2). We obtained 58.2% coverage of the IKKε protein and 64.7% (22 out of 34) coverage of the internal lysines. From this analysis, three lysine residues were identified as modified by ubiquitin: K30, K401, and K416.



1	MQSTANYLWH	TDDLLGQGAT	ASVY**K**ARN**K**K	SGELVAV**K**VF	NTTSYLRPRE
51	VQVREFEVLR	**K**LNHQNIV**K**L	FAVEETGGSR	Q**K**VLVMEYCS	SGSLLSVLES
101	PENAFGLPED	EFLVVLRCVV	AGMNHLRENG	IVHRDI**K**PGN	IMRLVGEEGQ
151	SIY**K**LTDFGA	ARELDDDE**K**F	VSVYGTEEYL	HPDMYERAVL	R**K**PQQ**K**AFGV
201	TVDLWSIGVT	LYHAATGSLP	FIPFGGPRRN	**K**EIMYRITTE	**K**PAGAIAGAQ
251	RRENGPLEWS	YTLPITCQLS	LGLQSQLVPI	LANILEVEQA	**K**CWGFDQFFA
301	ETSDILQRVV	VHVFSLSQAV	LHHIYIHAHN	TIAIFQEAVH	**K**QTSVAPRHQ
351	EYLFEGHLCV	LEPSVSAQHI	AHTTASSPLT	LFSTAIP**K**GL	AFRDPALDVP
401	**K**FVP**K**VDLQA	DYNTA**K**GVLG	AGYQALRLAR	ALLDGQELMF	RGLHWVMEVL
451	QATCRRTLEV	ARTSLLYLSS	SLGTERFSSV	AGTPEIQEL**K**	AAAELRSRLR
501	TLAEVLSRCS	QNITETQESL	SSLNRELV**K**S	RDQVHEDRSI	QQIQCCLD**K**M
551	NFIY**K**QF**KK**S	RMRPGLGYNE	EQIH**K**LD**K**VN	FSHLA**K**RLLQ	VFQEECVQ**K**Y
601	QASLVTHG**K**R	MRVVHETRNH	LRLVGCSVAA	CNTEAQGVQE	SLS**K**LLEELS
651	HQLLQDRA**K**G	AQASPPPIAP	YPSPTR**K**DLL	LHMQELCEGM	**K**LLASDLLDN
701	NRIIERLNRV	PAPPDV			

Figure 2. Mass spectrometry of IKKε.
(Left) GST-IKKε and HA-ubiquitin (HA-Ub) were coexpressed in HEK293T cells and lysates from 45 x 10 cm plates were loaded on SDS-PAGE and stained with Coomassie Blue. The bands noted by asterisks were submitted for mass spectrometry (Right) Summary of mass spectrometry data. Underlined amino acids were identified by mass spectrometry. Lysines (K) are bolded and the three lysines in red were found to be ubiquitinated.

Specific Aim 1c: I have generated site-specific lysine-to-alanine IKKε mutants for the three lysine residues that were identified in Aim 1b. These IKKε mutants have been retrovirally introduced into HA1EM cell to create stable cell lines that express these mutant constructs. These mutants were assessed for transformation capacity by both soft agar analysis (Figure 3a) as well as injection into immunocompromised mice (Figure 3b). From this analysis, I have determined that the K30 and K401 residues are essential for IKKε-mediated transformation. However, it appears that mutation of the K416 residue does not perturb the transformation phenotype of IKKε.

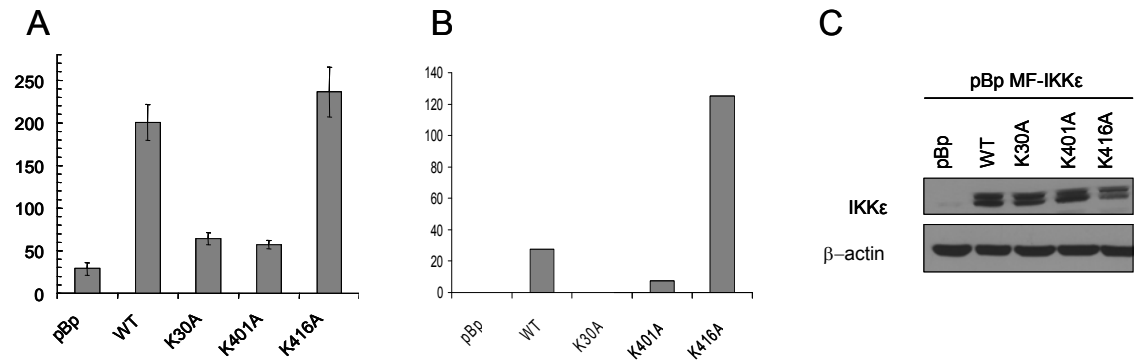


Figure 3. IKKε mutations perturb transformation phenotype.
(A) Soft agar analysis of HA1EM cells expressing WT and mutants of MF-IKKε. Colonies were counted 3 weeks after cells were plated.
(B) Tumor volume analysis of HA1EM cells expressing WT and mutants of MF-IKKε, injected into immuno-compromised mice. Each cell line was injected in 6 sites, $2x10^6$ cells per site. Tumor dimensions were measured 6 weeks after injection.
(C) Western blot analysis to show expression levels of IKKε in the cells plated in part (A) and (B)

Ongoing Studies: Having established that IKKε is modified by ubiquitination, my next aim will be to characterize what type of ubiquitination is occurring. It has been well-established that there are two types of ubiquitin modifications that both play important roles in the NF-κB pathway – K48- and K63- linked ubiquitination. My preliminary studies of IKKε show that proteosome inhibitor treatment does not affect the levels of IKKε protein expression (Figure 4a), this indicates that IKKε is undergoing a form of non-degradative ubiquitin modification. I, next, cotransfected mutant HA-ubiquitin constructs, in which all other lysines except K48 or K63 are replaced with Arg, with MF-IKKε into 293T cells. I immunoprecipitated for IKKε using M2 Flag affinity sepharose and immunoblotted for HA (Figure 4b). These results indicate that IKKε is subject to modification by both K48- and K63- linked ubiquitination.

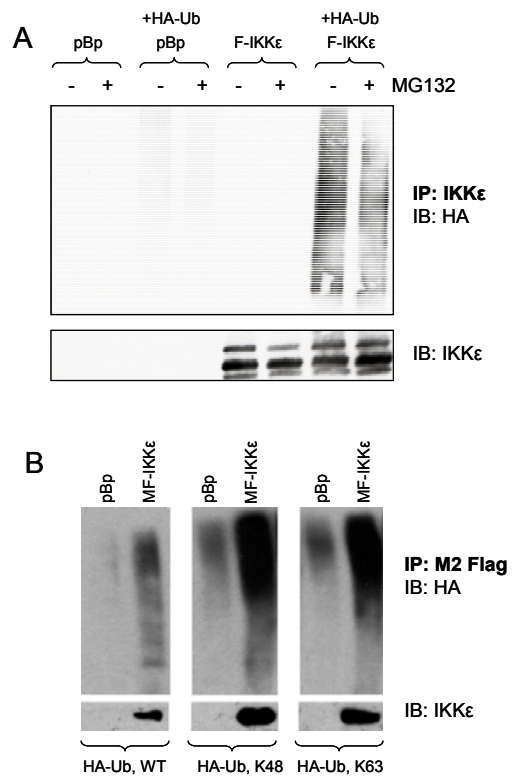


Figure 4. IKKε is modified by both K48- and K63-ubiquitn.

(A) 293T cells were cotransfected by F-IKKε and HA-Ub. Lysates were immunoprecipitated by the IKKε antibody and immunoblotted for HA.
(B) 293T cells were co-transfected by MF-IKKε and HA-Ub, wildtype and K48- or K63- only ubiquitin. Lysates were immunoprecipitated by M2 Flag sepharose and immunoblotted for HA.

My next step will be to determine what type of modification is occurring at each of the residues within IKKε that were identified in Aim 1b. I intend to do this by using a combination of the IKKε mutants generated in Aim 1c and the HA-ubiquitin mutants that were described above. In addition, I would like to determine the functional consequences of IKKε ubiquitination. To assess this, I will perform IKKε kinase assays to determine if IKKε kinase activity is affected, as well as look at the induction of well-known NF-κB downstream effectors to assess the effects on NF-κB activation.

Finally, since it is well-known that the IKK proteins act in large multi-protein complexes, I would like to determine the binding partners of IKKε in the context of mammary cell transformation. In collaboration with the CeMM Laboratory in Vienna, Austria, we will use mass spectrometry techniques to determine the protein binding partners of wild-type and mutant IKKε in the context of cell transformation.

Specific Aim 2a: I have successfully generated a MMTV-IKKε transgenic mouse model. By genotyping analysis, 9 founder mice were identified. These founders were bred out and assessed for mammary gland-specific expression of IKKε by immunoblot (Figure 5). From this analysis, 2 founder lines with varying levels of IKKε expression were chosen and are currently being expanded and followed. In addition, all of the original founder mice were retained and monitored. We currently have a cohort of 23 MMTV-IKKε mice of varying ages, the oldest of which are ~18 months in age (Figure 6, top). In addition, 3 of the original founder female mice have died from natural causes at an average age of ~20 months, no mammary tumor phenotypes have yet been observed in any of the MMTV-IKKε mice. Those founder lines that have been expanded were also bred to MMTV-ErbB2 mice, which are known to have a delayed mammary gland tumor formation phenotype. These MMTV-IKKε/MMTV-ErbB2 bitransgenic mice were observed to determine if there is a synergistic tumor formation phenotype. Our data, so far, show that IKKε does not cooperate with ErbB2 to accelerate tumor formation (Figure 6, bottom). This result is somewhat expected because IKKε and ErbB2 are effectors of the same pathway and, therefore, we would not expect them to synergize to accelerate tumor formation.

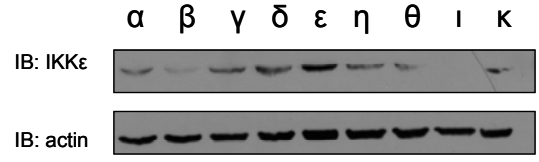


Figure 5. Analysis of MMTV-IKKε founder lines.
9 IKKε founder lines were established and analyzed for mammary gland specific IKKε expression by Western blot.

	AGE (months)				
	0~6	**6~12**	**12~18**	**18~**	***TOTAL***
MMTV-IKBKE	0	12	10	1	***23***
WT	0	18	4	0	***22***
TOTAL	***0***	***30***	***14***	***1***	***45***

	n	**% Tumor**	**Time to Tumor**
MMTV-IKBKE/ErbB2	10	90	~38 weeks
MMTV-ErbB2	4	100	~35 weeks

Figure 6. IKKε transgenic mice do not show tumor phenotype.
(Top) Current inventory of MMTV-IKKε mouse cohorts; so far, no mice have shown a mammary tumor phenotype.
(Bottom) Current inventory of MMTV-IKKε/ErbB2 compound transgnic mouse cohort; the compound transgenic does not show a significant acceleration in tumor formation timeline.

We have also obtained WAP-Cre and p53 floxed mice from the Mouse Models of Human Cancer Consortium (MMHCC) and are now in the process of crossing the MMTV-IKKε mice into a mammary-specific p53 null background. Our preliminary results in soft agar have shown that IKKε alone can induce colony formation in the mouse fibroblast cell line NIH3T3. These cells are p53 deficient, which provides good support that the introduction of IKKε in the context of p53 deficiency will result in cooperativity and an accelerated tumor formation phenotype. Currently, we have a total of 55 WAP-Cre/p53floxed double positive mice (Figure 7) and are in the process of breeding them with the MMTV-IKKε mice.

	AGE (months)			
FEMALE	**0~3**	**3~6**	**6~**	***TOTAL***
WAP-Cre/p53 floxed	20	8	0	***28***
WAP-Cre	6	0	0	***6***
p53 floxed	8	0	2	***10***
WT	1	0	0	***1***
TOTAL	***35***	***8***	***2***	***45***

	AGE (months)			
MALE	**0~3**	**3~6**	**6~**	***TOTAL***
WAP-Cre/p53 floxed	16	10	1	***27***
WAP-Cre	3	0	1	***4***
p53 floxed	7	0	0	***7***
WT	2	0	0	***2***
TOTAL	***28***	***10***	***2***	***40***

Figure 7. Current cohort of WAP-Cre/p53 floxed mice.

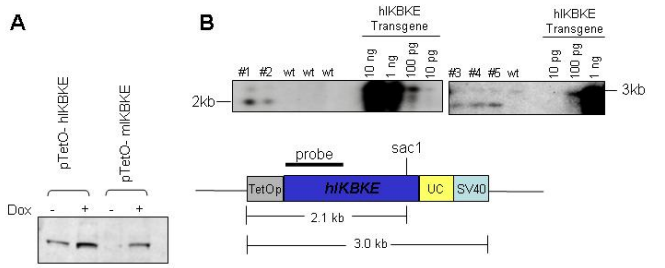


Figure 8. Inducible *hIKBKE* transgenic founder mice established
(A) NIH3T3 cells that stably express rtTA are transfected with the inducible transgenic construct pTetO-hIKBKE (human cDNA) and pTetO-mIKBKE (mouse cDNA). Cells were treated with 10μg/ml doxycycline for 24 hours. Immunoblot analysis (WB) on lysates was performed with an IKKε antibody
(B) Genomic DNA was made from tails of putative transgenic mouse pups and digested with SacI. Southern genotyping analysis was performed using a probe that hybridizes to the 3' region of the human *IKBKE* transgene. Linearized hIKBKE transgene was used at varying DNA concentrations as a control. Five founder mice were genotyped and contain the hIKBKE transgene in varying copy numbers.

Specific Aim 2b: We have successfully generated a doxycycline-inducible MMTV-rtTA/TetO-IKKε (MTB/TO-IKKε) bitransgenic mouse model. By genotyping analysis, 5 founder mice were identified (Figure 8). After analysis for doxycycline-inducible mammary gland IKKε expression, 2 founder lines with varying levels of IKKε expression were chosen and are currently being expanded and followed.

We currently have 40 female MTB/TO-IKKε mice (Figure 9a). All MTB/TO-IKKε bitransgenic mice are put on a doxycycline diet starting at the age of 3 weeks and have since remained on this diet. We have assessed for IKKε expression at three timepoints thus far: immediately after genotyping analysis (3 weeks age), 21 days after doxycycline diet (6 weeks age) and finally, at the sacrifice date of the oldest mice (17 months of age). We have observed robust and specific transgene expression in these mice, all the way through to the final timpoint (Figure 9b). Thus far, these mice have not yet shown a tumor formation phenotype. Therefore, we are also currently breeding them into the WAP-Cre/p53 floxed line to determine if IKKε expression will accelerate tumor formation in a mammary-specific p53 deficient background.

A

	AGE (months)				
FEMALE	**0~6**	**6~12**	**12~18**	**18~**	***TOTAL***
MTB/TO-IKBKE	10	8	14	8	***40***
TO-IKBKE	5	1	2	0	***8***
MTB	1	5	4	2	***12***
WT	2	1	7	2	***12***
TOTAL	***18***	***15***	***27***	***12***	***72***

B

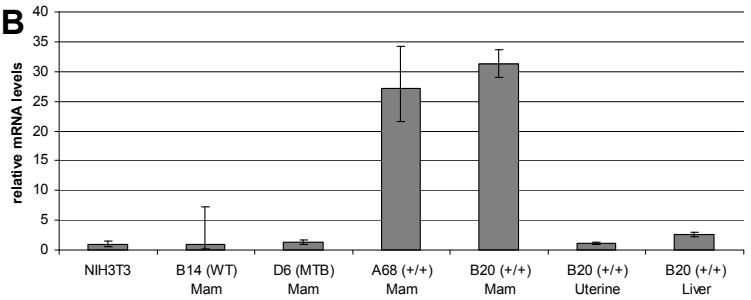


Figure 9. Current cohort of MTB/TO-IKKε mice.
(A) Current inventory of MTB/TO-IKKε mouse cohorts, all mice have been on doxycycline treatment since 3 weeks of age. So far, no mice have shown a mammary tumor phenotype.
(B) Real-time PCR analysis of IKBKE transgene expression at ~17 months of age, all +/+ have been on doxycycline treatment since 3 weeks of age

Key Research/Training Accomplishments

- Confirmed IKKε ubiquitination in the context of mammary cell transformation
- Identified three lysine residues within IKKε that are subject to ubiquitination by mass spectrometry
- Generated IKKε point mutants in which the identified lysine residues are mutated
- Generated cell lines that stably express the IKKε mutant constructs
- Demonstrated that IKKε lysine mutants show a distinct change in transformation phenotype *in vitro* and *in vivo*

- Generated founder mice for the constitutive MMTV-IKKε transgenic mouse model
- Generated founder mice for the inducible MMTV-rtTA/TetO-IKKε transgenic mouse model
- Generated compound transgenic MMTV-IKKε/MMTV-ErbB2 mouse model
- Generated compound transgenic MMTV-IKKε/WAP-Cre/p53$^{fl/fl}$ mouse model

Reportable Outcomes

- Developed HA1EM mutant IKKε cell lines: both F-IKKε and MF-IKKε with the following mutations K30A, K401A, K416A
- Developed four new animal models: MMTV-IKKε and MMTV-rtTA/TetO-IKKε, MMTV-IKKε/MMTV-ErbB2, MMTV-IKKε/WAP-Cre/p53$^{fl/fl}$

Conclusion

Over the course of the pas two years, I have made significant progress in all aspects of my Specific Aims as laid out in my original Statement of Work.

I have been able to identify three residues of IKKε that undergo modification by ubiquitination, and I have been able to show that the mutation of these residues is able to perturb the IKKε-mediated transformation phenotype. However, my mutational studies have also indicated that there is a more complex and dynamic role for IKKε ubiquitination than I had originally hypothesized. I believe that IKKε is undergoing a combination of both Lys48- and Lys63- linked ubiquitination, and that this combination of modifications is serving to tightly regulate both the levels of IKKε protein in the cell as well as its enzymatic activation. I propose to do a series of IP/Western experiments that utilize the Lys48- and Lys63- only ubiquitin mutants in combination with the IKKε mutants, in order to determine the exact nature of these modifications. In addition, to understand how the perturbation of the various residues of IKKε affects its function, I will assay these mutants for IKKε kinase activity as well as NF-κB activation. Finally, to understand how IKKε functions in complex to mediate cell transformation, in collaboration with the CeMM Laboratories, I will determine the protein binding partners of both wildtype and mutant IKKε in the context of cell transformation using a mass spectrometry approach.

I have also successfully generated both a constitutive and inducible transgenic mouse model of mammary-specific IKKε expression. Thus far, these mice have not shown a robust tumor formation phenotype. In parallel, I have bred the constitutive MMTV-IKKε with MMTV-ErbB2 mice to determine if there is a synergistic tumor formation phenotype – these mice, thus far, have also not shown any tumor acceleration. Therefore, as originally laid out in my Statement of Work proposal, I am currently working to generate a cohort of WAP-Cre/p53 floxed and will cross the MMTV-IKKε mice into this mammary-specific p53 null background to determine of IKKε promotes tumor formation/acceleration in a p53 deficient background.